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Excretion of Neutral α-Glucosidase, Determined with a Continuous Assay, and of Acid α-Glucosidase in the Urine of Human Reference Subjects

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Summary: The catalytic activities of neutral and acid α-glucosidase were selectively determined in human urine. Urinary excretion of neutral and acid α-glucosidase in reference subjects was found to be in the range 1.61 to 20.36 μkat/mol creatinine and 7.47 to 33.60 μkat/mol creatinine, respectively. Urinary excretion of both enzymes was not related to sex, age or diuresis. A continuous assay was introduced to improve the determination of neutral α-glucosidase.

Bestimmung der Ausscheidung von neutraler α-Glucosidase mit einem kontinuierlichen optischen Test und von saurer α-Glucosidase im Urin von Referenzpersonen

Zusammenfassung: Die Aktivität von neutraler und saurer α-Glucosidase wurde selektiv im menschlichen Urin bestimmt. Im Urin von Referenzpersonen wurde eine Ausscheidung von neutraler α-Glucosidase zwischen 1,61 und 20,36 μkat/mol Kreatinin und von saurer α-Glucosidase zwischen 7.47 und 33,60 μkat/mol Kreatinin gemessen. Die Ausscheidung beider Enzyme war weder geschlechts-, alters- noch diureseabhängig. Zur Optimierung der Bestimmung von neutraler α-Glucosidase wird ein kontinuierlicher optischer Test eingeführt.

Introduction

Human urine contains activities of neutral α-glucosidase (EC 3.2.1.20, α-*D*-glucoside glucohydrolase) as well as of acid α-glucosidase (EC 3.2.1.3, 1,4-α-*D*-glucan glucohydrolase) (1–4). Due to the extremely low activity of both enzymes in human serum (5–9) and to the high molecular weight of α-glucosidases (10–13) it is reasonable to assume that neither α-glucosidases are filtered to a significant extent in the renal glomerulus. The activity of neutral α-glucosidase originates from brush border membranes of renal proximal cells (14), whereas acid α-glucosidase originates from lysosomes (15). Human urinary excretion of α-glucosidase has been measured in control subjects and in patients with various diseases (1–4, 16–28).

In the preceding paper we reported optimal test conditions for the selective determination of neutral and acid α-glucosidase, using discontinuous assays (29). In the present investigation we have determined the urinary excretion of both enzymes in 24-h urines of reference subjects. These data may serve as controls for future studies on urinary excretion of neutral and acid α-glucosidase in patients. The determination of neutral α-glucosidase was improved by introducing a continuous assay.

Materials and Methods

Reagents

ATP, $NADP^+$, hexokinase (ATP: *D*-hexose-6-phosphate phosphotransferase (EC 2.7.1.1)) and glucose-6-phosphate dehydrogenase (*D*-glucose-6-phosphate: $NADP^+$ 1-oxidoreductase (EC 1.1.1.49)) were purchased from Boehringer, Mannheim, Germany; bovine serum albumin (99%) and *D*(+)-turanose were from SERVA, Heidelberg, Germany, and Multistix® from Miles, Ames Division, Frankfurt/M., Germany. All other chemicals were analytical grade from Merck, Darmstadt, Germany.

Subjects

To determine the activity of neutral α-glucosidase 150 urine samples (collecting period 24 h) were studied, including 90 specimens from apparently healthy subjects who were allowed to engage in their normal activities and to maintain their usual fluid intake, except alcohol, and 60 specimens from control patients. 101 specimens were collected for the determination of urinary acid α-glucosidase, 77 samples from apparently healthy subjects and 24 samples from control patients. In all apparently healthy subjects the urinalysis was normal. All control patients were admitted to the hospital for fractures, inguinal hernia, piles or uncomplicated gallstone disease; apart from the current complaint, they were apparently healthy, as judged from physical examination and routine laboratory tests, including serum creatinine determination. No subject had a medical history of metabolic diseases or diseases of the kidnies and urinary tract, and none was using drugs known to affect enzyme excretion in urine. The age of all subjects ranged between 10 and 82 years.

Pretreated urines

24-h urines were collected in polythene bottles containing sodium azide (final concentration about 1 g/l) to prevent bacterial growth. The volume was measured and aliquots of the native urines were subjected to urinalysis. 10 ml of native urine were centrifuged at 1200 *g* for 5 min to sediment cells and debris. The top 4 ml of urine were mixed with 1 ml bovine serum albumin to achieve an albumin concentration of 2 g/l. This sample was concentrated (about 2-fold) by ultrafiltration and subsequently diafiltered using a modified ultrafiltration system (Multi-Micro Ultrafiltration System MMCA consisting of 8 individual cells supplied with YM 10 Diaflo® membranes, both from Amicon, Witten, Germany). Ultrafiltration was performed using a pressure of 127.9 kPa (1.3 bar); subsequent diafiltration was performed for 30 min using the same pressure and sodium chloride solution (150 mmol/l) to separate urinary α-glucosidase from low molecular weight substances which may interfere with the enzyme determination. After diafiltration the volume of each ultrafiltration cell was stirred for 15 min to remove substances retained on the filter. After collection of the pretreated urine sample, 146.4 ± 18.3 µl ($n = 8$) remained in each cell of the modified ultrafiltration system as dead space. The average recovery of the activities of neutral and acid α-glucosidase in this procedure (corrected for dead space) was 97.6 ± 1.8% and 97.3 ± 1.9%, respectively. Albumin concentrations in the cell (before ultrafiltration) of less than 1 g/l showed lower recoveries, albumin concentrations of 2 to 4 g/l showed constant recoveries. Loss of glucose during the procedure was determined using 5 ml samples with added glucose (2, 5 and 100 mmol/l); 96.9 to 97.7% of the glucose was removed. 0.1 ml aliquots of the pretreated urines were used for the determination of neutral or acid α-glucosidase.

Assays

Continuous assay for neutral α-glucosidase

To improve our assay for a selective determination of neutral α-glucosidase (29), the enzyme activity was assayed in a continuous optical test with hexokinase as auxiliary enzyme and glucose-6-phosphate dehydrogenase as indicator enzyme (30), according to the following equations:

Tab. 1. Continuous assay of neutral α-glucosidase (EC 3.2.1.20).

Final concentrations in the test		
Phosphate buffer pH 7.0	80	mmol/l
Magnesium sulphate	5	mmol/l
$NADP^+$	2.5	mmol/l
ATP	2.5	mmol/l
Maltose	20	mmol/l
D(+)-Turanose	25	mmol/l
Hexokinase	50	µkat/l
Glucose-6-phosphate dehydrogenase	80	µkat/l

As shown later in the "Results and Discussion", the following assay conditions were optimal for the continuous assay of neutral α-glucosidase (tab. 1). The enzyme reactions were started by the addition of a 0.1 ml aliquot of a pretreated urine sample to 0.9 ml reagent mixture. The time-dependent changes in NADPH absorbance at 334 nm at 25 °C were registered automatically in intervals of 3 min with an Eppendorf Digitalphotometer 6118 fitted with cuvette changer 2705 and printer 6522 (Eppendorf Gerätebau Netheler & Hinz, Hamburg, Germany), or with a PMQ 3 Photometer (Zeiss, Oberkochen, Germany) connected to a Metrawatt RE 511 recorder. Pretreated urine samples were measured in triplicate. Three blank samples (150 mmol/l sodium chloride containing 2 g/l bovine serum albumin) were used as controls, and in all determinations the absorbance of the reagent mixture was registered for two 10 s periods before the addition of the 0.1 ml samples. After the lag phase, at least three time-dependent changes in NADPH absorbance were used to calculate the activities of neutral α-glucosidase, expressed in nmoles maltose hydrolysed per second per liter (nkat/l) according to the following equation:

Catalytic activity concentration

$$= \frac{1}{2} \times \frac{\Delta A}{\Delta t} \times \frac{v_2 \times 1.25}{v_1} \times \frac{V}{\varepsilon \times L \times v}$$

$$= \frac{\Delta A}{3\ \text{min}} \times \frac{v_2 \times 1.25}{v_1} \times 4494.8\ [\text{nkat/l}],$$

where $\Delta A/\Delta t$ is the change in absorbance per time interval between two measurements; since 2 mol glucose are liberated per 1 mol maltose the absorbance must be divided by 2; $\frac{v_2 \times 1.25}{v_1}$ is the urine pretreatment factor (v_1 is the volume in the ultrafiltration cell before ultrafiltration, v_2 is the volume in the ultrafiltration cell after ultrafiltration and diafiltration and 1.25 is the urine sample dilution factor caused by mixing with albumin solution); V is the reaction volume in the cuvette (1.0 ml), ε is the molar lineic absorbance (618 $m^2 \times mol^{-1}$), L is the pathlength of the cuvette (0.01 m) and v is the aliquot of pretreated urine sample used (0.1 ml).

The urinary activity of α-glucosidase was expressed in nkat/24 h and in µkat/mol urinary creatinine.

The reagent mixture for the continuous assay of neutral α-glucosidase is stable for at least 3 days at 4 °C.

$$\text{Maltose} + H_2O \xrightarrow[(EC\ 3.2.1.20)]{\text{Neutral α-glucosidase}} 2D\text{-Glucose}$$

$$2\ D\text{-Glucose} + 2\ ATP \xrightarrow[(EC\ 2.7.1.1)]{\text{Hexokinase}} 2D\text{-Glucose-6-}P + 2\ ADP$$

$$2\ D\text{-Glucose-6-}P + 2\ NADP^+ \xrightarrow[(EC\ 1.1.1.49)]{\text{Glucose-6-}P\text{ dehydrogenase}} 2D\text{-Gluconolactone-6-}P + 2\ NADPH + 2\ H^+$$

The activity of acid α-glucosidase was determined by a two point method (29). Creatinine concentration was measured enzymatically (31) and urinary protein concentration was determined with bovine serum albumin as the standard (32).

Statistics

Data on urinary excretion of neutral and acid α-glucosidase revealed a lognormal distribution according to the fractile graphical analysis (33). Using logarithmically transformed values, normal limits (2.5, 50 and 97.5 percentiles) were calculated. Sex-related differences in our experimental data were evaluated using the t-test for heterogeneous variances, and age-related differences were evaluated by calculating the coefficient of linear correlation.

Results and Discussion

Continuous assay of neutral α-glucosidase

By using discontinuous assays we have achieved selectivity for the determination of neutral and acid α-glucosidase (29). Several advantages such as rapidity and accuracy led us to the development of the continuous assay for neutral α-glucosidase described in this paper. The activity of neutral α-glucosidase was highest at pH 7.0 to 7.2 (fig. 1) in a phosphate buffer at a concentration of 80 mmol/l (fig. 2). The catalytic rate of reaction showed saturation at ATP and $NADP^+$ concentrations of 1.5 mmol/l (fig. 3). ATP and $NADP^+$ were used at somewhat higher concentration (2.5 mmol/l) in the test. To determine an optimal concentration for the auxiliary and indicator enzymes, absorbance differences were measured at varying hexokinase activity concentrations (15 to 80 μkat/l) at a glucose-6-phosphate dehydrogenase activity concentration of 40 μkat/l; and at varying glucose-6-phosphate dehydrogenase activity concentrations (15 to 80 μkat/l) at a hexokinase activity concentration of 40 μkat/l. Under these conditions the reaction rate was constant, but the lag phase increased with decreasing catalytic concentrations of glucose-6-phosphate dehydrogenase. Therefore a hexokinase activity concentration of 50 μkat/l and a glucose-6-phosphate dehydrogenase activity concentration of 80 μkat/l was chosen. The relation between the activity of neutral α-glucosidase and substrate turnover was linear (fig. 4) in the range from 1 to 2000 nkat/l. The reaction rate was proportional to the α-glucosidase concentration. The lag phase decreased slightly with increasing α-glucosidase concentration. The average lag phase was 2.48 ± 0.84 min (n = 28). A linear relation was also found by comparing the results of the discontinuous and the continuous method with a somewhat higher recovery of 106.2 ± 7.5% for the latter (fig. 5). Using the continuous assay, the K_m-value of neutral α-glucosi-

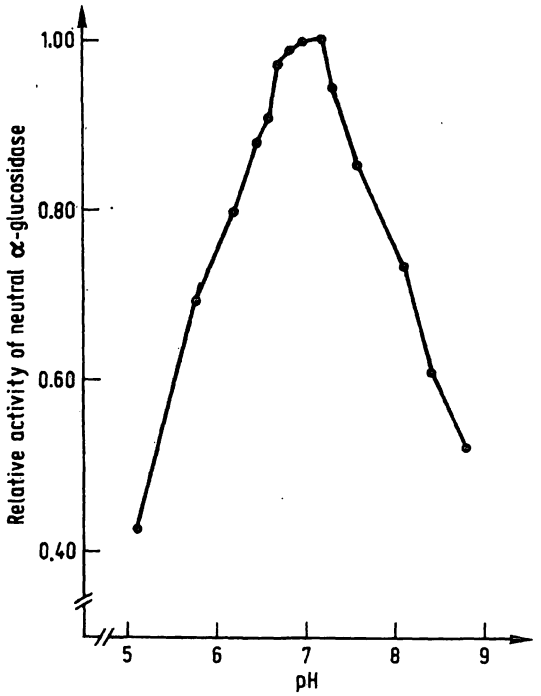

Fig. 1. Relative activity of neutral α-glucosidase as a function of pH. All other conditions were held constant.

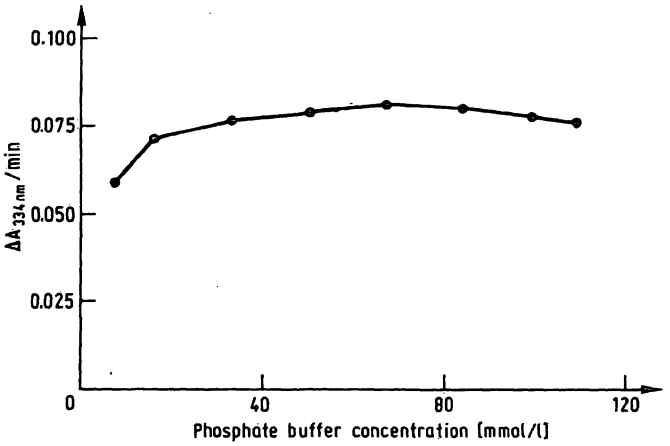

Fig. 2. Rate of change of absorbance as a function of phosphate buffer concentration. All other conditions were held constant.

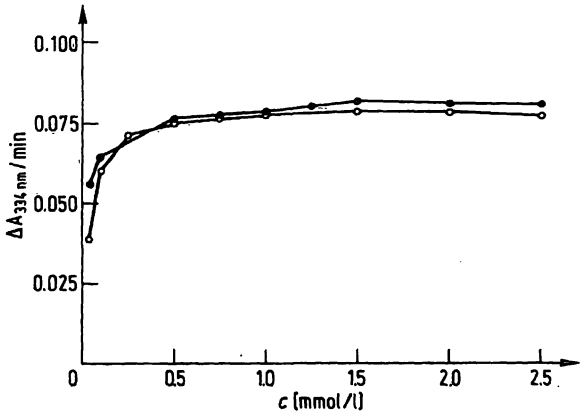

Fig. 3. Rate of changes of absorbance as a function of ATP (O——O) concentration at a $NADP^+$ concentration of 1.5 mmol/l and as a function of $NADP^+$ (●——●) concentration at an ATP concentration of 1.5 mmol/l. All other conditions were held constant.

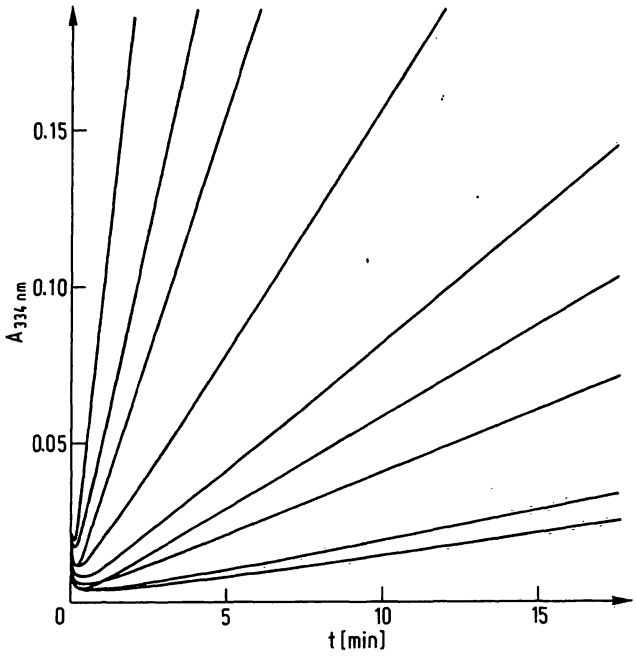

Fig. 4. Original graphs of absorbance as a function of time measured at various catalytic activity concentrations of α-glucosidase (1360, 680, 453, 227, 110, 80, 55, 26 and 18.5 nkat/l). All other conditions were held constant, except that measurements were performed at 37 °C.

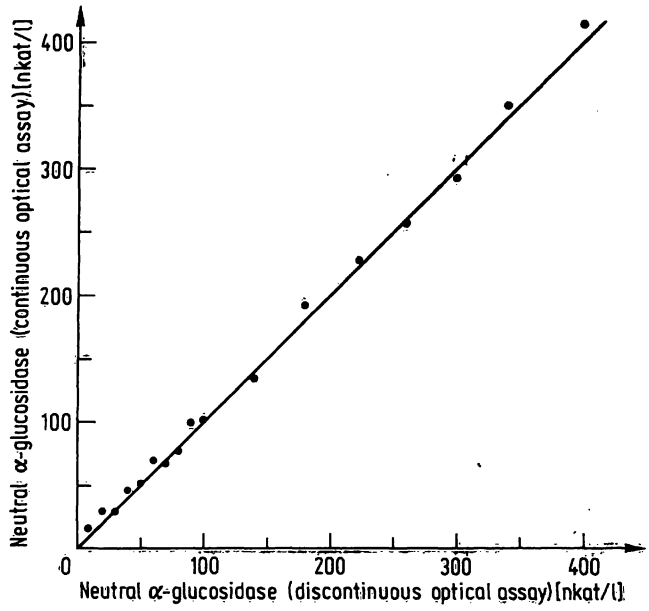

Fig. 5. Comparison of measurement of catalytic activity concentrations of neutral α-glucosidase performed by continuous and discontinuous optical assay, —— regression line y = x.

Tab. 2. Statistical evaluation of the urinary excretion of neutral α-glucosidase in female and male healthy subjects[a]) and control patients[b]). Estimates of normal limits (2.5, 50 and 97.5 percentiles) and ranges are given for: enzyme catalytic concentration, enzyme excretion per 24 h and per mol creatinine, urine volume per time and creatinine concentration.

	Sex	Percentiles			Range	Unit
		2.5	50	97.5		
			Healthy subjects			
Neutral α-glucosidase	♀	15.7	48.7	151	13.3 – 178	nkat/l
	♂	20.7	59.6	171	8.7 – 178	
	♀	20.0	54.0	146	16.8 – 195	nkat/24 h
	♂	26.1	75.1	216	13.0 – 213	
	♀	1.82	5.00	13.69	1.81 – 12.22	μkat/mol
	♂	2.06	5.83	16.74	1.61 – 20.36	creatinine
Urine volume	♀	0.56	1.11	2.20	0.60 – 2.20	l/24 h
	♂	0.59	1.30	2.72	0.40 – 2.30	
Creatinine	♀	4.60	9.81	20.86	3.98 – 17.86	mmol/l
	♂	4.77	10.25	22.28	4.77 – 23.69	
			Control patients			
Neutral α-glucosidase	♀	15.8	50.7	162	13.3 – 152	nkat/l
	♂	16.8	56.6	190	19.3 – 221	
	♀	24.3	63.5	167	23.3 – 113	nkat/24 h
	♂	18.9	72.3	277	10.7 – 213	
	♀	2.65	6.88	17.87	2.68 – 12.78	μkat/mol
	♂	2.68	6.62	16.29	2.15 – 13.35	creatinine
Urine volume	♀	0.64	1.25	2.46	0.70 – 2.15	l/24 h
	♂	0.65	1.34	2.66	0.55 – 2.15	
Creatinine	♀	4.60	7.34	24.58	4.42 – 19.27	mmol/l
	♂	3.89	8.66	19.54	3.54 – 16.80	

[a]) 90 healthy subjects, 50 males with a mean age of 36 years, 40 females with a mean age of 41 years.
[b]) 60 control patients, 34 males with a mean age of 42 years, 26 females with a mean age of 59 years.

dase for maltose (K_m = 0.67 mmol/l) and the inhibitor constant for turanose (K_I = 5.2 mmol/l) were determined. Both constants and the type of inhibition (competitive) are in agreement with those obtained by the discontinuous assay (29).

The within-run precision of our continuous method was assayed by a series of 20 determinations of the activity of neutral α-glucosidase of the same specimen. The mean value was 109.5 nkat/l, with a coefficient of variation of 2.1% (± 2.3 nkat/l). The day-to-day precision was studied for 10 days by repeated analysis of aliquots of the same specimen. The coefficient of variation was 4.4%.

Albumin concentrations up to 8 g/l in pretreated urine samples had no effect on the determination of neutral α-glucosidase. Glucose concentrations up to 1.5 mmol/l affected only the lag phase in pretreated urine samples.

The activity of neutral α-glucosidase is inhibited by turanose, potassium ions, tris and erythritol (see l.c. (29)). In the discontinuous assay for neutral α-glucosidase (29) and in the continuous assay described here, turanose has no effect on the activity of neutral α-glucosidase, but inhibits almost completely the residual activity of acid α-glucosidase (29).

Neutral as well as acid α-glucosidase were found to be stable for several days at 4 °C and even at room temperature in native urine samples. Three urine samples stored at −20 °C for as long as 6 months showed less than 10% loss of activity of neutral and acid α-glucosidase, respectively.

Urinary excretion of neutral and acid α-glucosidase

Urinary excretion of activities of neutral as well as acid α-glucosidase in female and male healthy subjects and control patients, estimates of normal limits and ranges and experimental details are compiled in table 2 and 3. No statistically significant differences could be detected, except that urinary excretion of both enzymes per 24-h is higher in men than in

Tab. 3. Statistical evaluation of the urinary excretion of acid α-glucosidase in female and male healthy subjects[a]) and control patients[b]). Estimates of normal limits (2.5, 50 and 97.5 percentiles) and ranges are given for: enzyme concentration, enzyme excretion per 24 h and per mol creatinine, urine volume per time and creatinine concentration.

	Sex	Percentiles			Range	Unit
		2.5	50	97.5		
		Healthy subjects				
Acid α-glucosidase	♀	64.4	150	350	66.8 −332	nkat/l
	♂	73.2	166	381	78.3 −355	
	♀	67.4	160	378	81.6 −335	nkat/24 h
	♂	112	217	423	88.3 −440	
	♀	6.82	16.40	39.37	7.84− 33.60	μkat/mol
	♂	6.93	16.18	37.90	7.47− 33.60	creatinine
Urine volume	♀	0.49	1.08	2.40	0.50− 2.20	l/24 h
	♂	0.67	1.30	2.52	0.55− 2.30	
Creatinine	♀	3.98	9.02	21.22	4.07− 17.86	mmol/l
	♂	4.51	10.25	23.16	4.42− 23.69	
		Control patients				
Acid α-glucosidase	♀	39.0	121	378	55.0 −396	nkat/l
	♂	55.6	160	462	48.3 −290	
	♀	54.8	152	422	46.0 −320	nkat/24 h
	♂	60.7	195	630	48.3 −461	
	♀	8.26	15.50	28.96	8.29− 26.58	μkat/mol
	♂	9.23	15.50	26.02	10.94− 28.62	creatinine
Urine volume	♀	0.58	1.26	2.68	0.70− 2.15	l/24 h
	♂	0.74	1.22	2.02	0.80− 1.80	
Creatinine	♀	3.27	7.87	19.01	4.60− 19.27	mmol/l
	♂	4.86	10.34	22.10	4.42− 16.80	

[a]) 77 healthy subjects, 41 males with a mean age of 41 years, 36 females with a mean age of 47 years.
[b]) 24 control patients, 10 males with a mean age of 46 years, 14 females with a mean age of 58 years.

women in both healthy subjects and control patients. The same sex-related difference in urinary excretion of both enzymes was also found ($P < 0.001$) when both groups of healthy subjects and control patients were taken together. At all levels tested urinary excretion of both α-glucosidases was not related to age or diuresis. Thus for the evaluation of urinary excretion of both α-glucosidases, females and males of both groups of healthy subjects and control patients were taken together to represent the reference group, and the urinary excretion was considered exclusively in μkat per mol urinary creatinine. These reference subjects excreted renal brush border neutral α-glucosidase in a relatively small range from 1.61 to 20.36 μkat/mol creatinine (n = 150) and renal lysosomal acid α-glucosidase in a somewhat broader range from 7.47 to 33.60 μkat/mol creatinine (n = 101). Statistical data are compiled in table 4.

Tab. 4. Statistical evaluation of the urinary excretion of neutral α-glucosidase per mol creatinine for all subjects investigated of both sexes (150 control subjects) and the corresponding values for acid α-glucosidase (101 control subjects). Estimates of normal limits (2.5, 50 and 97.5 percentiles) and ranges are given.

Percentiles			Range
2.5	50	97.5	
Neutral α-glucosidase (μkat/mol creatinine)			
2.17	5.93	16.20	1.61–20.36
Acid α-glucosidase (μkat/mol creatinine)			
7.25	15.97	35.67	7.47–33.60

Intra-individual comparison of the excreted activities of neutral and acid α-glucosidase showed that the activity of acid α-glucosidase was always higher than that of neutral α-glucosidase (n = 79). The most frequent ratio was between 2 and 3, with an asymmetric distribution characterized by the following normal limits: ratio 1.2 (2.5 percentile), ratio 2.9 (50 percentile), and ratio 6.7 (97.5 percentile).

The day-to-day variation of urinary excretion of neutral and acid α-glucosidase per mol urinary creatinine was very small, i.e. 7.5 and 6.5%, respectively. Diurnal variations in the urinary excretion of neutral and acid α-glucosidase was observed throughout a 24-h period by collecting urine at 3-h intervals. As shown earlier for the urinary α-glucosidase excretion measured at pH 5.0 (24), the urinary excretion of neutral and acid α-glucosidase was high between 3 a.m. and 9 a.m. and low during the afternoon and evening. Thus urines for the determination of both α-glucosidases should be collected at fixed time intervals, preferably during a 24-h period.

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